Journal of Ceramic Processing Research. Vol. 18, No. 11, pp. 796~800 (2017)

J O U R N A L O F
Ceramic
Processing Research

Processing and properties of biodegradable BCP/Collagen membrane.

Woo Seong Tak[a], Yu Sang Choi[b] and Su Chak Ryu[c,*]

[a]*Department of Nano Fusion Technology, Pusan National University, Korea*
[b]*Bio Team, Dentis Co., Ltd., Korea*
[c]*Department of Nano Mechatronics Engineering, Pusan National University, Korea*

Biphasic calcium phosphate (BCP) is a bioceramic that has two phases of tricalcium phosphate and hydroxyapatite which is inorganics composing bone. That has both osteoconductive and osteoinductive properties. Collagen is organic component composing bone. The aim of this paper is an assessment about membrane that was made with biphasic calcium phosphate and collagen. The specimens have various ratios of BCP/Collagen. The main production process is dry-freezing. It was undergone for 48 hrs. The specimens were evaluated for morphology, chemical behavior, cell cytotoxicity and animal test. The morphology was observed by scanning electron microscope. About the chemical behavior, specimens were measured using inductively coupled plasma optical emission spectrometer at 4 weeks and 8 weeks. Cell cytotoxicity was detected at 450 nm. For animal tests, rats were sacrificed at 12 weeks and 20 weeks.

Key words: BCP, Collagen barrier, Cell cytotoxicity, Rats, Animal test.

Introduction

Bone is a composite that made of ceramics and organic or inorganic materials. Those are collagen, hydroxyapatite (HAp) and many things. Bone plays the role of mechanical things that assist movement as well as metabolic function. If it makes a loss or defects, the body recovers parts of loss. However, its pace is rather slow. These matters give difficulties patients while the body recovers self. If patients hurt same parts again during recovery, it makes slower a recovery pace. These things make researcher study ways of recovering bone faster.

Calcium phosphate material is ceramics which compose of bone, and there are several sorts of calcium phosphate [1]. Of that, hydroxyapatite is most similar to bone structurally, chemically [1]. It has a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$. Many researchers have already investigated that hydroxyapatite have well-biocompatibility [1, 2]. That synthetic HAp as bone can be replacement in bio application is proven widely [2]. These assure HAp is well bone substitute, but its recover pace does not satisfy what pace people want. What has sufficient pace that can satisfy people is tricalcium phosphate [3, 4]. Tricalcium phosphate is also a calcium phosphate material and bone substitute. Unlike HAp, it degrades rather fast in the body. What has these properties is biphasic calcium phosphate (BCP). The mixing makes absorption pace faster than when HAp is used alone [3]. Tricalcium phosphate is osteoinductive and HAp is osteoconductive. The suitable ratio of HAp/TCP is 80/20 [3, 5]. Thereby, BCP has both osteoconductive and osteoinductive properties [6].

Collagen membrane can do a role as a carrier of BCP and barricade epithelium physically [7-9]. As bone consists of collagen, collagen does not cause an immune reaction and absorb into tissue. It has an excellent biocompatibility and induces new bone formation [10-12]. There are two ways to make collagen membrane. They are cross-linked and non-linked collagen membrane. The cross-linked collagen membrane is more resistant and affects the degree of resorption [13, 14]. Collagen membrane have a layer structure, from this property it can control the rates of absorbability and residual collagen [15]. According to how much layer and how strong crosslinking, rates of absorbing collagen and BCP can be adjusted [13-15].

Based on the above matters, BCP/Collagen membrane can assist to recovering bone. Moreover, it can be effective in dental fields due to the protection and recovery. On this paper, BCP/Collagen experimented on morphology, in vitro, chemical behavior and in vivo test.

Experimental

Preparation of BCP nano-sol

45 um under BCP powder (Dentis, Daegu, Republic of Korea), distilled water and ZrO_2 ball were mixed in ratio 1 : 1 : 4. The mixed solution was milled using

*Corresponding author:
Tel : +82-51-510-6112
Fax: +82-51-514-2358
E-mail: scryu@pusan.ac.kr

attrition mill equipment for 4 h at 500 rpm. Through a particle size analyzer (ELS-Z, Otsuka Electronics Co., Ltd, Japan), the particle size of BCP sol was measured and the result was 600 nm.

Preparation of BCP/Collagen membrane

1.0 wt% collagen solution was made by stirring distilled water and collagen (SK Bioland, Republic of Korea) for 2 hrs. BCP sol was added to collagen solution as 30, 50, 70 and 100 wt% to collagen. Each sample was designated as specimen D, specimen C, specimen B, and specimen A. BCP/Collagen solution was stirred for 2 hrs. The solution was frozen at −77 °C deep freezer for 2 hrs. Then, it was under freeze-drying for 48 hrs at −93 °C under 400 mTorr using freeze dryer (Frexi-Dry Mp, USA). The created membrane was pressed at 10 ton for 10 s using a hydraulic press. To crosslink collagen, it was in an ultraviolet sterilizer (Vision Scientific Co., Ltd, Republic of Korea) for 1hr per one side. In order that neutralizes the membrane, it was soaked in 0.1 M NaOH solution for 30 min and solution was eliminated. Then, it was dipped in distilled water for 15 min. The above two process was repeated five times. Then it was frozen for 2 hrs, and it was under freeze-drying for 24 hrs. It was pressed at 10 ton for 10 sec again.

SEM of BCP/Collagen Membrane

The morphology of the specimens was observed using SEM (AIS 2300C, Seron Tech, uiwang, Republic of Korea). Before detecting, the specimens were coated with gold.

Elution of BCP/Collagen membrane in SBF

60 cm^2 of the specimen was dipped in 10 ml of simulated body fluid (SBF). It was placed in a 37 °C incubator. At 4 weeks and 8 weeks, 1 ml of the supernatant was taken biweekly and centrifuged at 1500 rpm for 3 min to taking residue away from the supernatant. It was diluted with distilled water to 2% solution. The concentration of Ca and P ions was determined by ICP-OES (iCAP-6000, Thermo Scientific Inc, USA).

In vitro cytotoxicity

Culture medium was made to mix Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA), 10% fetal bovine serum (SAFC, USA) and 1% penicillin-streptomycin (Gibco). MG-63 cells (KCLB, Republic of Korea) were cultured in the culture medium, and the cell suspension was made at 1 × 105/ml concentration. 100 μl of the cell suspension was seeded into each well of the 96-well dish and placed in 37 °C and 5% CO_2 incubator (World Science, Republic of Korea) for 24 hrs. To make test solution, negative control, positive control and control group, 30 cm^2 of specimen, 30 cm^2 of high density polypropylene (Hatano Research Institution, Japan) and 0.5 g of natural rubber latex (Microflex, Belgium) was soaked in 5 ml of culture medium in conical tube respectively and 10 ml of culture medium was put in conical tube. All of the conical tubes was placed in a 37 °C incubator for 24 hrs. All medium in the 96-well dish was eliminated and changed to test solution, negative control, positive control, and control group. The 96-well dishes were placed in 37 °C and 5% CO_2 incubator for 24 hrs. 100ul of EZ-cytox (DoGenBio. Co., Ltd, Republic of Korea) was added to each well. The 96-well dishes were placed in 37 °C and 5% CO_2 incubator for 1h. Then, absorbance was detected at 450 nm using multilabel plate reader (Victor3, PerkinElmer, USA). The experiment refers to international standard [16].

In vivo animal experiment

Each rat was gotten an intramuscular injection with ketamine hydrochloride of 70 mg/kg concentration. After they had been cut fur, they were disinfected with povidone iodine and were fixed. The surgical parts were under infiltration anesthesia with 2% lidocaine which has 1 : 80,000 epinephrine and there were back subcutaneous incisions at three regions of 15mm intervals. So that avoids membranes bending by tissue, tissue was excoriated sufficiently. Then, each membrane of 10 × 5 mm was implanted on hypodermis. The parts were sutured with 4-0 monosyn, caring not to suture membrane and skin together. At 12 weeks and 20 weeks, the surgical parts were cut, they were observed. All process of the animal experiment proceeded in Pusan National University Laboratory Animal Resources Center, and the protocol was approved by Pusan National University Institutional Animal Care and Use Committee.

Results and Discussion

Morphology observation of BCP/Collagen membrane

The morphology of the specimens was shown in Fig. 1. Although collagen membrane has porous and web structure originally, the specimens have a shape weighed down and possessing a little of the pore. It is considered that this is because of the process that the specimens were pressed with force corresponding 10 ton. It can be discovered that collagen tangle together, it results from ultraviolet crosslinking. Owing to that phenomenon, it is expected that there is an improvement of resistance for resolving. It is inferred that BCI/Collagen membrane resolves rather slowly in the body. The large point of difference of ratio BCP/Collagen is not detected. It is just the matter of quantity of BCP. There were rather large BCP particles. It is aggregation which was formed in term between stirring and deep freezing. It is regarded as the cause of different densities between collagen and BCP and aggregation of BCP during stirring. The BCP particles are inside collagen. It means that the particles do not expose until

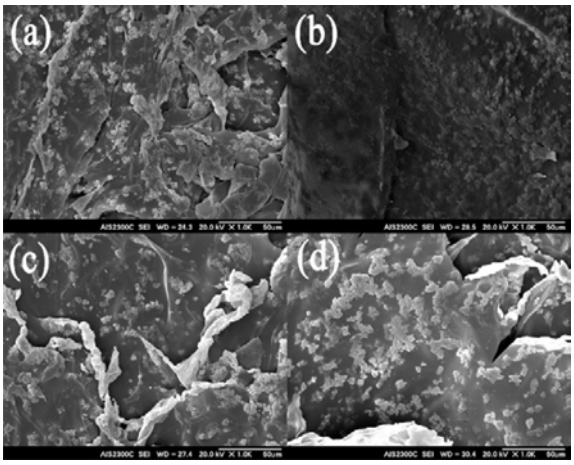

Fig. 1. SEM images of the BCP/Collagen membranes' surface. (a), (b), (c) and (d) are specimen A (BCP: collagen weight ratio = 1 : 1), B (0.3 : 1), C (0.5 : 1) and D (0.3 : 1) respectively.

collagen dissolve. If body fluid enters inside the membrane, the particles can approach fluid and dissolve. However, because the specimens were pressed, the surface area became smaller, and the dissolution pace of BCP/Collagen membrane became slow down.

Chemical behavior of BCP/Collagen membrane

Fig. 2 show the concentration of Ca and P ion at 4 weeks and 8 weeks. The BCP/Collagen weight ratio of specimen A, B, C, and D were 1, 0.7, 0.5 and 0.3. In all kinds of the samples, the concentration of Ca and P ion at 8weeks increased as compared with 4weeks. As times goes on, the collagen membrane was melting. It exposed BCP particles to SBF, and the particles dissolve. The pace of collagen melting was increased gradually. However, it was decreased entering 8weeks. The more content of BCP was little in the samples, the more phenomenon was gotten intense. The reason for this phenomenon is assumed that an absolute quantity of BCP lessened and the pace of dissolving also became slow. In the case of P ion, the less the specimen has the amount of BCP, the less result shows the concentration of P ion. The degree is bigger as the ratio of BCP is lower. These behaviors mean BCP and collagen have no difference of dissolving pace. It is

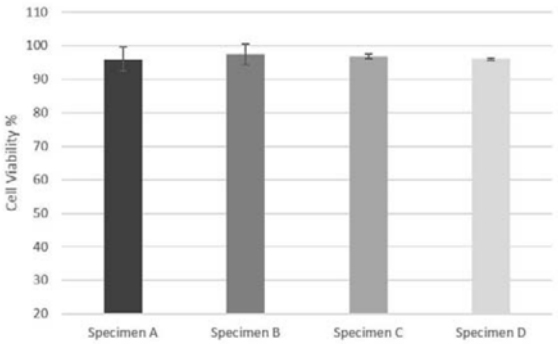

Fig. 3. Cell viability of BCP/Collagen membrane. The average result of control group's cell viability was designated to 100%.

inferred that BCP and collagen hold each other and the holding is strong, so they dissolve simultaneously in the specimens. Therefore, the less the specimen has the quantity of BCP, the slower the dissolving pace.

Cytotoxicity of BCP/Collagen membrane

The used EZ-cytox of the experiment is measured at 450 nm. About the samples, they have a purpose of assisting bone recovery, so MG-63 cell was used. The cell line was osteoblast and fibroblast. In the measurement, the average absorbance of control group over 0.2 and the average's difference between two lines of the control group in the 96-well dish was under 15%. Also, the cell viability of negative control was over 70%, and the positive control was under 70%. Therefore, the error of experiment was regarded as no exist. About four ratios of the specimens, the difference of cell viability is not shown largely, and all of the specimens show over 90% viability. (Fig. 3) According to evaluation method, the specimens have no cytotoxicity 16.

In vivo assessment of BCP/Collagen membrane

Fig. 4 show the insertion parts of BCP/Collagen membrane. The rats were sacrificed at 12 weeks and 20 weeks. Some case revealed that the particle does not absorb to tissue yet. In the case of the specimen A, the sample at 12 weeks remained approximately 10% BCP particle. At 20 weeks, BCP dissolved and absorbed absolutely. About the sample C, the samples at 12

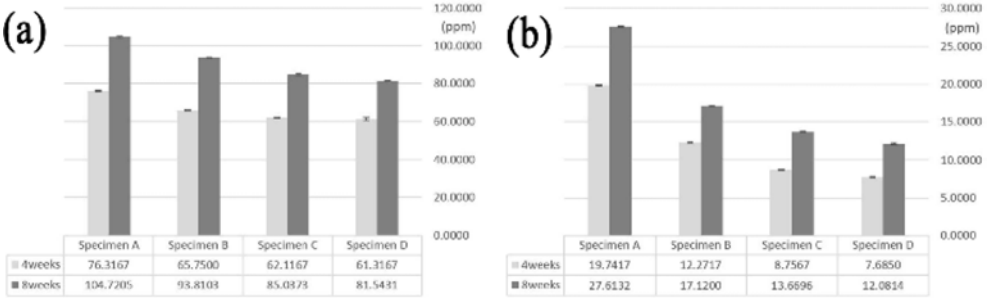

Fig. 2. Chemical behavior of BCP/Collagen membrane in SBF. Concentration of (a) Ca and (b) P with four kinds of weight ratio.

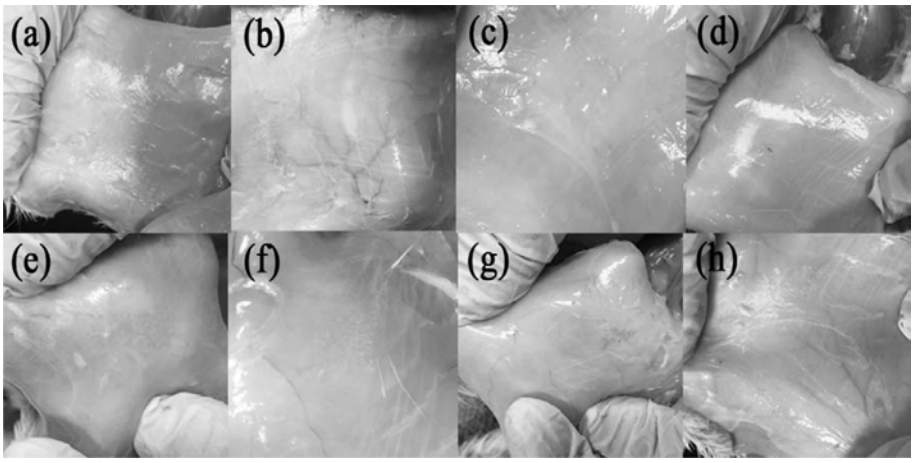

Fig. 4. Photograph of surgical regions. (a), (b) were inserted with specimen A. (c), (d) and (e) were inserted with specimen C. (f), (g) and (h) were inserted with specimen D. (a), (c), (d), (f) and (g) were sacrificed at 12 weeks. (b), (e) and (h) were sacrificed at 20 weeks.

weeks remained 20% and 10% BCP. The sample at 20 weeks even remained 20% BCP. About the sample D, the samples at 12 weeks showed the remains of 30% BCP and the sample at 20 weeks showed the remains of 20% BCP. The less the specimens have BCP particles, the much the samples have BCP. It means that the BCP/Collagen membrane has low absorption pace as it has little BCP. It is considered that collagen linked BCP showed fast melting pace firstly and then remained BCP has lower melting pace because BCP and collagen interact each other for melting pace. The specimens did not show any immune reaction like edema and red spots. Exceptively, blood vessel kinking phenomenon appeared at sample D at 12 weeks. It implied that excessive regeneration of tissue occurred and unusual shape was showed.

Conclusions

The BCP/Collagen membranes have the morphology that collagen linked together and BCP particle positioned in the intervals of collagen. The collagen showed some morphology of bulk. It is considered this is made during the process of UV-crosslinking. The difference does not exist about ration of BCP. In the process, the BCP particle aggregate together. Originally, the BCP sol has a 600 nm particle size, but the SEM result showed bigger BCP particles. It affects the pace of absorption, and it is not considered of big effects because BCP has a fast pace. In SBF, BCP/Collagen membranes have unusual things. The less the specimens have BCP, the slower the specimens have the dissolution pace. Collagen and BCP have high biocompatibility and, even tissue composes of collagen, so the specimens have no cell cytotoxicity regardless of BCP ratio. All of the cell viability ran to 100% approximately. In in vivo test, rat's skin layer showed that BCP particles do not remain much and it dissolved almost. One case showed the blood vessel kinking phenomenon. It is an abnormal phenomenon because of excessive regeneration. Overall data suggests deeper experiments about the interaction between BCP and collagen. Unlike conventional chemical thought, some different data was showed.

Acknowledgments

This work (Grants No. S2332433) was supported by the Project of Technology Development funded Korea Small and Medium Business Administration in 2015.

References

1. W.G. Billotte, in "Biomaterials: principles and applications" (CRC Press 2000) p.27.
2. M. Vallet-Regi, J. Chem. Soc. 2 (2001) 97-108.
3. Z. Zhang, H. Kurita, H. Kobayashi, and K. Kurashina, Oral Science International 2 (2005) 85-95.
4. C.P.A. T. Klein, A.A. Driessen, and K. de Groot J. Biomed. Mater. Res. 64 (1983) 769-784.
5. R.Z. Leegeros, S. Lin, R. Rohanizadeh, D. Mijares, and J.P. LeGeros, J. Mater. Sci. Mater. Med. 14 (2003) 201-209.
6. I. Manjubala, T.P. Sastry, J. Biomater. Appl. 19[4] (2005) 341-360.
7. L. Ronald, R.L. van Swol, R. Ellinger, J. Pfeifer, N.E. Barton, and N. Blumenthal, J. periodontal. 64[7] (1993) 622-629.
8. R. Dimitriou, G.I. Mataliotakis, G.M. Calori, and P.V. Giannoudis, BMC. Med. 10[81] (2012) 2-24.
9. T.P. Queiroz, E. Hochuli-Vieira, M.A. Gabrielli, and D.C. Cancian, Int. J. Oral. Maxillofac. Implants. 21[1] (2006) 29-35.
10. Y. Zubery, A. Goldlust, A. Alves, and E. Nir, J. Periodontol. 78[1] (2007) 112-121.
11. S. Kung, H. Devlin, E. Fu, K. Y.Ho, S. Y. Liang, and Y.D. Hsieh, J. Periodont. Res. 46 (2011) 126-133.
12. C.-K. Lee. K.-T. Koo, T.-I. Kim, Y.-J. Seol, Y.-M. Lee, I.-C. Rhyu, Y. Ku, C.-P. Chung, Y.-J. Park, and J.-Y. Lee, J. Periodontal. Implant. Sci. 40 (2010) 232-238.
13. H. Tal, A. Kozlovsky, Z. Artzi, C.E. Nemcovsky, and O. Moses, Clin. Oral. Implant. Res. 19[2] (2008) 295.
14. T. Kodama, M. Minabe, T. Hori, and Y. Watanabe, J.

Periodontology 60[4] (1989) 205.
15. H. Tal, O. Moses, A. Kozlovsky, and C. Nemcovsky. in "Bone regeneration", (Intech 2012) p.111.
16. ISO 10993-5:2009. Biological evaluation of medical devices- part 5: tests for in vitro cytotoxicity.